http://www.kidney-international.org

Transcription factor Ets-1 is essential for mesangial matrix remodeling

M Mizui[1], Y Isaka[1,2], Y Takabatake[1], Y Sato[3], H Kawachi[4], F Shimizu[4], S Takahara[2], T Ito[1] and E Imai[1]

[1]Department of Nephrology, Osaka University Graduate School of Medicine (A8), Suita, Japan; [2]Department of Advanced Technology for Transplantation, Osaka University Graduate School of Medicine (A8), Suita, Japan; [3]Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan and [4]Department of Cell Biology, Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

Most advanced glomerular diseases are characterized by abnormal extracellular matrix (ECM) accumulation in the glomeruli, and matrix metalloproteinases (MMPs) play a pivotal role in ECM remodeling in various glomerular diseases. The proto-oncogene, *ets-1*, is a transcription factor regulating the expression of various matrix proteinases, including MMP-1, MMP-3, and MMP-9. The goal of the present study was to characterize the role of Ets-1 in the progression of glomerular diseases. Overexpression of Ets-1 in cultured mesangial cells prevented transforming growth factor (TGF)-β-induced inhibition of DNA-binding activity and TGF-β-induced type I collagen production. In addition, exogenous Ets-1 abolished TGF-β-induced collagen gel contraction. The *in vivo* transfection of the *ets-1* gene into nephritic kidney resulted in the increases in glomerular MMP-1, MMP-3, and MMP-9 mRNA, decreases in mesangial ECM deposition, and attenuation of fibronectin extradomain A (EDA) and type I collagen expression. In contrast, knockdown of Ets-1 in glomeruli resulted in severe ECM deposition in diseased glomeruli. In conclusion, Ets-1 promotes degradation of ECM proteins and is critical for integral glomerular reorganization.

Kidney International (2006) **70,** 298–305. doi:10.1038/sj.ki.5001541; published online 31 May 2006

KEYWORDS: TGF-β; gene therapy; mesangial cells

Correspondence: *Y Isaka, Department of Advanced Technology for Transplantation, Osaka University Graduate School of Medicine (A8), Suita 565-0871, Japan. E-mail: isaka@att.med.osaka-u.ac.jp*

Received 6 December 2005; revised 6 February 2006; accepted 22 March 2006; published online 31 May 2006

Most advanced glomerular diseases are characterized by increased accumulation of extracellular matrix (ECM) in glomeruli. The normal glomerular ECM is primarily composed of type IV collagen, laminin, fibronectin, and sulfated proteoglycans, which are produced by mesangial cells and epithelial cells.[1,2] However, activated glomerular cells secrete these matrix components in excessive amounts and also secrete disease-specific matrix components, including type I and type III collagens.[3,4] Although the mechanisms of ECM accumulation are not clearly understood, studies suggest that ECM accumulation and progression to glomerulosclerosis is associated with an imbalance between synthesis and degradation of these matrix components.[5] Further, matrix metalloproteinase (MMP) may also play an important role in ECM remodeling in various renal diseases.

The transcription factor *ets-1* mediates embryogenesis, angiogenesis, cell migration, and invasion.[6–9] The Ets domain binds to the Ets-binding motif GGAA/T in the *cis*-acting element of target genes, and cooperates with the c-Fos/c-Jun complex at the AP-1 site to induce expression of several promoters.[10] This motif is also present in the promoter region of various other genes, including MMP-1, MMP-3, MMP-9, urokinase-type plasminogen activator, and tissue inhibitor of metalloproteinase-1.[11–16] Ets-1 is expressed in fibroblasts and endothelial cells[6,8,9,13] and is transiently expressed in endothelial cells during vascular development in the embryo and during angiogenesis in the adult. Further, inhibition of Ets-1 expression resulted in the inhibition of endothelial cell migration, invasion, and angiogenic activity.[17]

The *ets-1* gene also contributes to kidney development by activating forkhead-related transcription factor (FREAC), a winged helix transcriptional factor detected during nephrogenesis.[18] However, the role of the renal Ets-1 expression profile is controversial in the context of glomerular disease. Naito *et al.*[19] demonstrated that progression of rat crescentic glomerulonephritis was associated with a significant increase of Ets-1-positive cells, consisting mainly of epithelial and endothelial cells but not of mesangial cells. In contrast, Raffetseder *et al.*[20] identified mesangial cells as the main source of glomerular Ets-1 expression in the context of

ant-Thy-1 nephritis. Further, the temporal increase of mesangial Ets-1 expression correlated with mesangial cell activation in experimental glomerulonephritis.[20]

Based on these data, it is not clear whether Ets-1 acts as a protective regulator or as a progression factor for renal disease. Thus, the goal of the present study was to characterize the role of Ets-1 in the progression of glomerular diseases.

RESULTS

Ets-1 prevents TGF-β-induced effects on mesangial cells

By utilizing *ets-1*-transfectant mesangial cells, DNA-binding activity of a biotin-labeled double-stranded oligonucleotide harboring the Ets-1 consensus-binding sequence was assessed by electrophoretic mobility shift assay. As shown in Figure 1a, TGF-β1 inhibited binding of a distinct nucleoprotein in normal cultured mesangial cells in a dose-dependent manner. Overexpression of Ets-1 prevented the inhibitory effect of TGF-β1 on DNA binding.

In normal rat mesangial cells, treatment with TGF-β resulted in increased expression of type I collagen in a dose-dependent manner. However, overexpression of Ets-1 prevented this effect (Figure 1b). Further, overexpression of Ets-1 also reduced the basal levels of type I collagen, suggesting that the inhibitory effect of Ets-1 on ECM remodeling may occur via a TGF-β-independent mechanism as well as TGF-β-dependent pathway.

TGF-β1 significantly enhanced collagen gel contraction in normal mesangial cells but not in cells overexpressing Ets-1 (Figure 1c). It has been reported that TGF-β1 increases collagen matrix reorganization by increasing mesangial $\alpha 1\beta 1$ integrin.[21] These data suggest that overexpression of Ets-1 may prevent rearrangement of collagen matrix via inhibition of TGF-β signaling.

Exogenous Ets-1 induces MMP expression and activity

Ets-1 was expressed very low, supposedly in endothelial and epithelial cells, not in mesangial areas in normal kidney (Figure 2a), whereas Ets-1 expression was significantly increased not only in endothelial and epithelial cells but also in some mesangial cells 7 days after Thy-1 antibody injection (Figure 2b). We also investigated the glomerular *ets-1* and the matrix-related gene expressions in anti-Thy-1 glomerulonephritis. Reverse transcriptase-polymerase chain reaction (PCR) analysis showed that *ets-1* and tissue inhibitor of MMP-1 mRNA were increased on the day 3 and maintained even on day 28. Although MMP-2 was upregulated for 1 week and tapered by degrees, MMP-9, as well as MMP-1 and MMP-3 (data not shown), did not show significant changes in the course of anti-Thy-1 nephritis (Figure 2c). Mesangial cells express a number of proteases that degrade ECM proteins during glomerular matrix remodeling. Among them, not MMP-2 but MMP-1, MMP-3, and MMP-9 contain

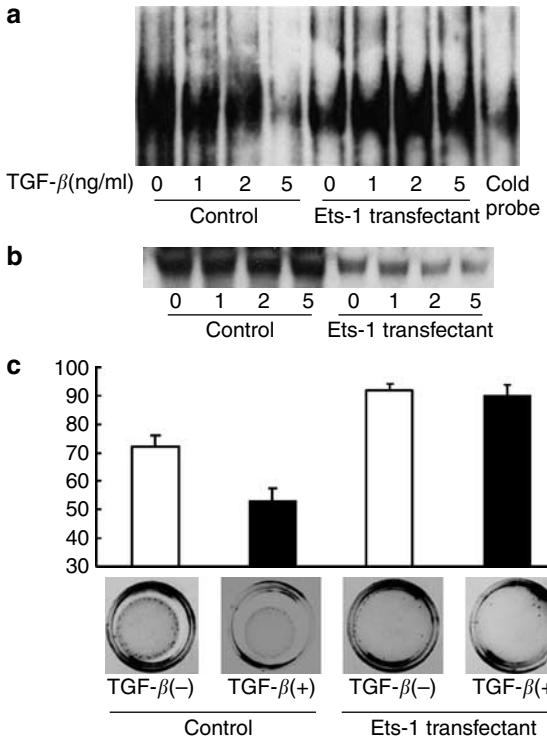

Figure 1 | Ets-1 overexpression in cultured mesangial cells. Rat mesangial cells were transfected with ets-1 cDNA, and established ets-1 permanent transfectants. (**a**) In normal rat mesangial cells, DNA-binding activity was reduced by TGF-β stimulation in a dose-dependent manner, but ets-1 transfectant maintained the DNA-binding activity. (**b**) In normal rat mesangial cells, the treatment with TGF-β increased the expression of type I collagen. In contrast, type I collagen expression was diminished below basal levels in *ets-1* transfectants. (**c**) Although TGF-β significantly enhanced collagen gel contraction in normal mesangial cells, gel contraction by *ets-1*-transfectant mesangial cells was almost abolished.

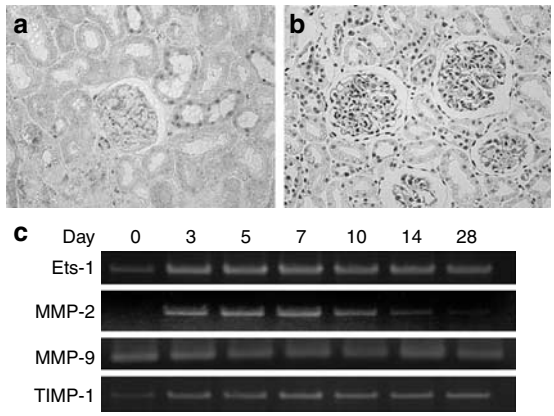

Figure 2 | Ets-1 and MMPs expression in nephritic glomeruli. (**a**) Immunostaining of Ets-1 revealed that Ets-1 expression was very low, supposedly in the nuclei of endothelial and epithelial cells, not in mesangial cells of normal kidney, (**b**) but significantly increased in the nuclei of not only endothelial and epithelial cells but also in mesangial cells 7 days after Thy-1 antibody injection. Original magnification $\times 100$. (**c**) PCR analysis showed that *ets-1* and tissue inhibitor of metalloproteinase-1 mRNA were increased on day 3 and maintained even on day 28. MMP-2 was upregulated for 1 week and tapered by degrees. MMP-9 did not show significant changes in the course of anti-Thy-1 nephritis.

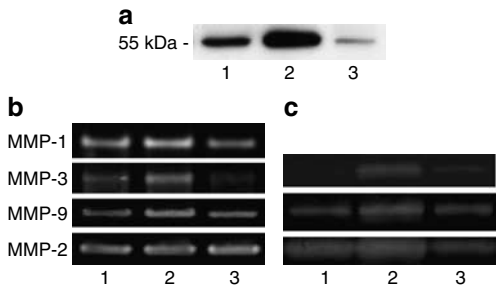

Figure 3 | Exogenous Ets-1 induces MMPs. (**a**) Western blot analysis demonstrated that ets-1-gene transfer (lane 2) markedly increased Ets-1 protein in glomeruli compared with untreated nephritic kidneys (lane 1) or contralateral right kidneys (lane 3). (**b**) Reverse transcriptase-PCR demonstrated that the levels of mRNA of MMP-1, MMP-3, and MMP-9 were markedly augmented in *ets-1*-gene transfected kidneys (lane 2) compared with those in untreated nephritic kidneys (lane 1) or untreated contralateral nephritic kidneys (lane 3). (**c**) Consistent with the results of (**b**), the glomerular activities of MMP-3 and MMP-9 in *ets-1*-tranfected kidney (lane 2) were more intense than those in untreated nephritic kidneys (lane 1) or untreated contralateral nephritic kidneys (lane 3).

Ets-1-binding motif in their *cis*-acting element. These results suggested that Ets-1 expression may not be sufficient in glomerular matrix remodeling.

To examine the effect of overexpression of ets-1 in mesangial cells, we transferred *ets-1* gene into the nephritic glomeruli by electroporation-mediated gene transfer, which enables us to introduce genes into glomerular mesangial cells.[22] As Western blot analysis demonstrated that *ets-1*-gene transfer markedly increased Ets-1 protein in glomeruli compared with contralateral right kidneys (Figure 3a), we next examined the effect of forced overexpression of Ets-1 on the ECM remodeling in nephritic glomeruli.

Reverse transcriptase-PCR demonstrated that the levels of mRNA of MMP-1, MMP-3, and MMP-9 were markedly augmented in kidneys transfected with *ets-1* compared with the untreated contralateral kidneys (3.3-fold increase in MMP-1, 5.2-fold in MMP-3, and 2.3-fold in MMP-9 compared with contralateral kidney) (Figure 3b). In contrast, the expression of MMP-2 mRNA was almost equal when comparing kidneys transfected with *ets-1* and the untreated contralateral kidneys.

MMP activity was assessed by glomerular gelatin zymography, which demonstrated that glomerular MMP-3 and MMP-9 activity was higher in kidneys transfected with *ets-1* compared with the untreated contralateral kidneys (4.8-fold increase in MMP-3 and 2.6-fold in MMP-9 compared with contralateral kidney) (Figure 3c). In contrast, activity of MMP-2 was equivalent when comparing the two groups.

Exogenous Ets-1 ameliorates histological changes

Histological analysis with periodic acid-Schiff staining showed marked ECM accumulation in the untreated contralateral kidneys (Figure 4a). In contrast, kidneys transfected with *ets-1* showed reduced ECM accumulation (Figure 4b). Glomerular matrix score was significantly

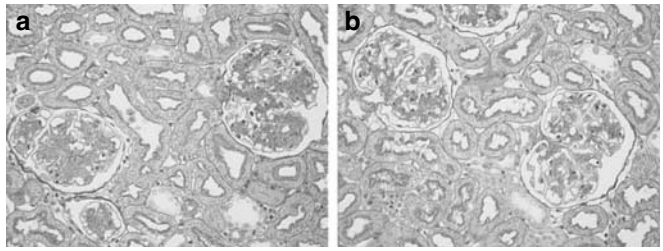

Figure 4 | Effect of Ets-1 on the progression of anti-Thy-1 glomerulonephritis on day 7. Micrographs show representative results of glomeruli stained with periodic acid-Schiff from untreated contralateral nephritic kidney (**a**) and *ets-1*-treated kidney (**b**) Original magnification $\times 200$.

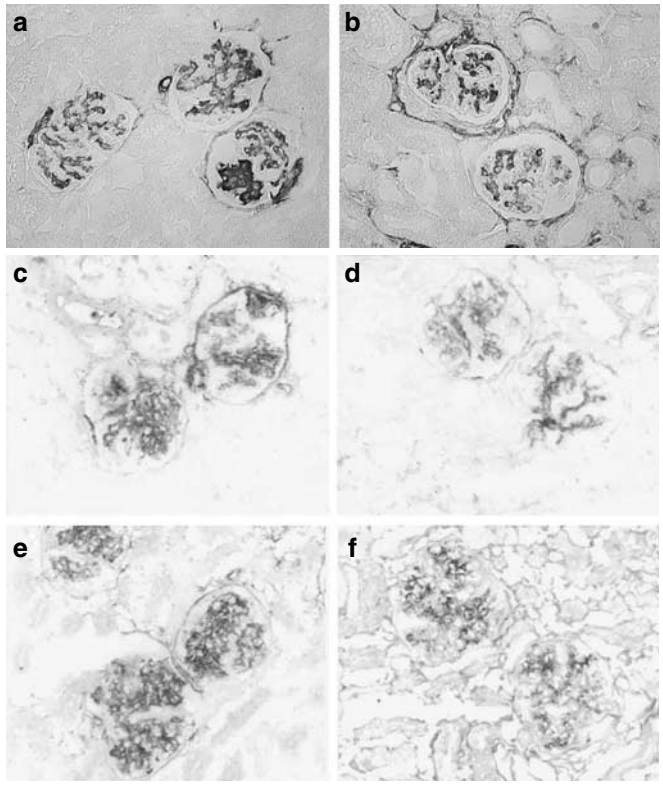

Figure 5 | Effect of Ets-1 on the ECM remodeling of anti-Thy-1 glomerulonephritis on day 7. Micrographs show representative results of glomeruli stained with (**a**, **b**) SMαA, (**c**, **d**) fibronectin EDA, and (**e**, **f**) type I collagen from (**a**, **c**, **e**) untreated contralateral nephritic kidney and (**b**, **d**, **f**) *ets-1*-treated kidney. Original magnification $\times 200$.

reduced in ets-1-treated left kidneys compared with untreated contralateral right kidneys (2.75 ± 0.68 and 1.88 ± 0.67, in untreated and *ets-1*-treated, respectively; $P < 0.01$). Further, expression of smooth muscle α actin (SMαA), fibronectin EDA, and type I collagen was much higher in the glomeruli of untreated kidneys (Figure 5a, c, e) than in *ets-1*-transfected kidneys (Figure 5b, d, f). Semi-quantitative analysis demonstrated that SMαA-positive areas were significantly decreased in *ets-1*-gene transfected group compared with untreated contralateral group (16.9 ± 5.6 and

24.7±5.8%, respectively; $P<0.01$). Similar observation was shown in fibronectin EDA (21.4±4.4 and 32.6±9.2; $P<0.001$) and type I collagen (20.7±4.4 and 39.1±8.7%; $P<0.001$) expression in *ets-1*-gene-transfected group compared with untreated contralateral grouprespectively. We also examined the involvement of ets-1 expression in normal glomeruli by ets-1 gene transfer. Ets-1 expression in normal glomeruli induced no significant histological changes (data not shown).

Ets-1 reduces ECM proteins in nephritic glomeruli

Western blot analysis demonstrated that glomerular fibronectin and type I collagen expression were increased in untreated nephritic kidney but not in *ets-1*-transfected kidneys (Figure 6). Laser densitometric analysis revealed that fibronectin and type I collagen expression in glomeruli from nephritic rats treated with ets-1 gene transfection was reduced to 20 and 48% of those in untreated disease control, respectively. These data suggest that Ets-1 is a potent inhibitor of ECM accumulation. Decreased levels of ECM proteins in glomeruli may result from the activation of the degradative pathways (e.g. MMP), decreased collagen synthesis, or both.

Knockdown of Ets-1 increases ECM deposition

Recent study demonstrated that the *ets-1* gene is essential for the normal development of mammalian kidneys and the maintenance of glomerular integrity and that the Ets-1 may act as an upstream regulator of the expression of FREAC-4 that is detected during kidney development. In the next step, we transferred short synthetic interfering RNA for *ets-1* (siEts-1) into the nephritic glomeruli to knockdown the *ets-1* expression in glomeruli. Compared with the nephritic kidney treated with siSCR (Figure 7), siEts-1-transfected nephritic kidney represents a marked ECM accumulation in glomeruli (Figure 3c) (2.58±0.87 and 3.42±0.73, in siSCR- and siEts-1-treated, respectively; $P<0.01$), suggesting that excessive ECM deposition progressed or ECM remodeling was delayed.

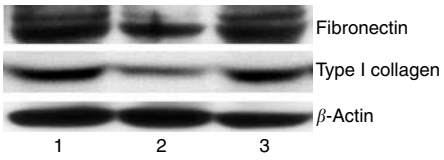

Figure 6 | Glomerular fibronectin and type I collagen from untreated nephritic kidneys (lane 1), *ets-1*-transferred kidneys (lane 2) or contralateral kidneys (lane 3) were analyzed by Western blot analysis. Glomerular fibronectin and type I collagen were increased in untreated nephritic kidney, but not in *ets-1*-transfected kidneys.

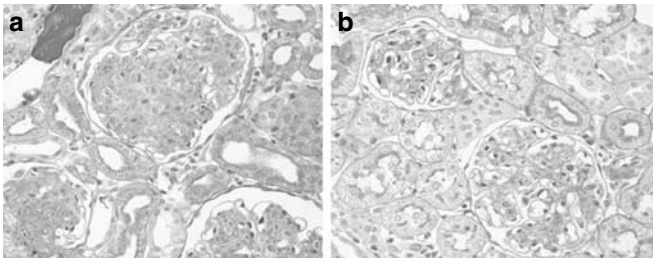

Figure 7 | Knockdown of Ets-1 increases ECM deposition. Morphological changes of a (**a**) siEts-1-treated or (**b**) siSCR-treated nephritic kidney. Representative microphotographs of glomeruli stained with periodic acid-Schiff were presented. Original magnification ×400.

DISCUSSION

The present study suggests that Ets-1 acts as a protective effector molecule and restores glomerular integrity. Overexpression of Ets-1 prevented the TGF-β-induced reduction in DNA-binding activity and prevented the TGF-β-induced increase in type I collagen production in cultured mesangial cells. In addition, exogenous *ets-1* inhibited collagen gel contraction induced by TGF-β, and *in vivo* transfection of *ets-1* into nephritic kidney resulted in increases in glomerular MMP-1, MMP-3, and MMP-9 mRNA. Finally, *ets-1*-transfected kidneys showed reduced mesangial ECM deposition and reduced fibronectin EDA and type I collagen expression, whereas knockdown of *ets-1* in glomeruli resulted in severe ECM deposition and diseased glomeruli.

The ECM is a network of macromolecules surrounding all cells and comprised of collagens, proteoglycans, and multiadhensive matrix proteins. Controlled ECM synthesis, deposition, and degradation occur during embryogenesis, angiogenesis, and tissue repair. In contrast, only limited turnover of the ECM occurs in the majority of normal adult tissues. However, the balance between ECM synthesis and degradation is disrupted in the context of various pathological conditions, leading to abnormal ECM remodeling. For example, excessive matrix synthesis and deposition is characteristic of fibrotic diseases, such as glomerulosclerosis. TGF-β is a potent renal fibrogenic growth factor[23] that upregulates ECM deposition via several different mechanisms, including stimulation of collagen synthesis, induction of the profibrotic cytokines, and inhibition of MMP and tissue inhibitor of metalloproteinase.[24]

In the present study, TGF-β reduced DNA-binding activity of Ets-1 in a dose-dependent manner in normal mesangial cells, but not in cells transfected with *ets-1*. In addition, overexpression of Ets-1 inhibited TGF-β-induced type I collagen production in cultured mesangial cells. Ets-1 interacts with the AP-1 (c-Fos/c-Jun) complex[25] to regulate various genes, including MMP-1 and MMP-3.[16] Further, elevated expression of Ets-1 in fibroblasts altered their responses to TGF-β in favor of matrix degradation relative to ECM deposition.[26] Furthermore, the antagonistic effect of Ets-1 against TGF-β1 was enhanced in the presence of TGF-β1.[26] We also examined the involvement of ets-1 expression in normal glomeruli by *ets-1* gene transfer. Transfection of *ets-1* gene into normal glomeruli induced no significant histological changes. Furthermore, *ets-1* gene transfection 7 days after disease induction induced no significant glomerular changes at day 14. Our findings that

forced expression of Ets-1 on non-injured normal glomeruli or later stage of self-limiting nephritic glomeruli induced no significant changes were consistent with the previous observations that Ets-1 induced MMPs activation were apparent in the presence of TGF-β1.[26] These data suggest that under pathological conditions, overexpression of Ets-1 may antagonize the profibrotic effects of TGF-β, possibly via AP-1-Ets-1-mediated prevention of TGF-β-Smad-dependent gene expression. Thus, we postulate that Ets-1 is a potent suppressor of TGF-β-induced genes. Of interest is that overexpression of Ets-1 significantly affect the basal levels of type I collagen, suggesting that the inhibitory effect of Ets-1 on ECM remodeling manifest itself in the context of TGF-β-independent signaling pathway, as well as TGF-β signaling pathway.

The present study demonstrated that exogenous Ets-1 prevented TGF-β-induced collagen gel contraction. The collagen contraction process is composed of several phases including cell migration, cell attachment to collagen fibers, and reorganization of the surrounding collagen fibers into a more dense and compact arrangement within the collagen lattice.[21] ECM remodeling requires proteases for matrix degradation as well as cell adhesion via integrins. The $\alpha1\beta1$ integrin is a collagen/laminin receptor that mediates collagen matrix reorganization in various organs.[21,27] TGF-β increases collagen matrix reorganization by increasing mesangial $\alpha1\beta1$ integrin, thereby leading to an increased adhesion of mesangial cells to type I collagen and promotion of glomerulosclerosis.[21] In addition, TGF-β upregulates tissue transglutaminase,[24] leading to further activation of TGF-β via crosslinking of the latent TGF-β-binding protein to the ECM.[28] Elevated levels of transglutaminase are associated with fibrotic diseases, where its ability to crosslink ECM may facilitate ECM deposition and promote resistance to the action of MMP.[29] Together with the present observations, these data indicate that overexpression of Ets-1 in mesangial cells may inhibit TGF-β-mediated mesangial cell association with collagen fibers, thereby leading to abnormal ECM reorganization.

Recently, it was reported that *in vivo* transfection of *ets-1* antisense ODN resulted in an inhibition of angiogenesis induced by the HGF gene in a rat ischemic hindlimb model.[30] We demonstrated that siEts-1-transfected nephritic kidney represents a marked ECM accumulation in glomeruli compared with the nephritic kidney treated with siSCR, suggesting that the inhibition of Ets-1 induced excessive ECM deposition or delayed ECM remodeling. Recent studies have demonstrated that Ets-1 is essential for the normal development of the mammalian kidneys and for maintenance of glomerular integrity. Further, Ets-1 may act as an upstream regulator of FREAC-4 expression during kidney development. Indeed, the Ets-1 knockout mice exhibits various glomerular abnormalities, including sclerosis, atrophy, and markedly fewer and immature glomeruli.[31] These results support a protective role for Ets-1 in preserving the normal structure of the kidney.

In order to prove the therapeutic effect of Ets-1 *in vivo* mesangial cells, we transferred *ets-1* gene into the mesangial cells of the nephritic glomeruli by electroporation. We demonstrated that *ets-1* gene transfer markedly increased Ets-1 protein in glomeruli, and thereby increased MMP expression and activity in glomeruli. In addition, SMαA, fibronectin EDA, and type I collagen expression was significantly suppressed in *ets-1*-transfected nephritic kidneys compared with untreated contralateral kidneys. Decreased levels of ECM proteins in glomeruli may result from the activation of the degradative pathways (e.g. MMP), decreased collagen synthesis, or both. In human glomerular diseases, increased TGF-β expression was observed in progressive glomerular diseases and there was strong correlation between the amount of TGF-β1 in biopsy and the degree of fibrosis.[23] TGF-β upregulates the synthesis of individual matrix components, including proteoglycans, collagens, and glycoproteins.[32] TGF-β also inhibits matrix degradation by increasing the synthesis of protease inhibitors and decreasing the synthesis of proteases.[33] And TGF-β alters the expression of integrins and modulates their relative proportions on the cell surface in a manner that could facilitate adhesion to the matrix.[34] These evidences strongly suggest that the inhibition of mesangial TGF-β expression should be one of the crucial therapeutic strategies to prevent the progression of renal fibrosis. Together with our *in vitro* experiments, our *in vivo* experiments suggest that forced overexpression of Ets-1 in glomeruli can suppress the ECM proteins by blocking TGF-β pathways. Furthermore, we demonstrated that *ets-1* transfection induced glomerular MMP-1, MMP-3, and MMP-9, which could degrade ECM proteins.

Vascular endothelial growth factor and basic fibroblast growth factor are angiogenic factors that induce expression of Ets-1 in endothelial cells.[13,35] Hepatocyte growth factor (HGF), a potent angiogenic and antifibrotic factor, also stimulated the expression of MMP-1 via induction of Ets-1 in endothelial cells and vascular smooth muscle cells.[30] Ets-1 plays an important role in angiogenesis by regulating the expression of MMP and urokinase-type plasminogen activator[13,17,36] and converting the endothelial cells to the angiogenic phenotype via inducing the expression of integrin $\beta3$.[7] As cell migration is a multistep process that involves adhesion, motility, and degradation of ECM, the migratory cells may be regulated by several stimuli during the tissue remodeling process. Mesangial cells were also demonstrated to migrate and repopulate the glomerulus in a coordinated manner after mesangial injury.[37] Vascular endothelial growth factor [38] and HGF[39] are reported to have therapeutic roles in various renal diseases. Of interest is that glomerular proliferation was also inhibited in ets-1-transfected kidney. Ets-1 was reported to have versatile effects on endothelial cells during the course of angiogenesis; that is, one is for cellular invasion, and the other is for the localized apoptosis.[40] This apoptotic effect of Ets-1 might inhibit the glomerular proliferation. Taken together with our report, the

induction of Ets-1 in mesangial cells may play a central role in mesangial migration and remodeling.

These data indicate that forced expression of Ets-1 is a potent inhibitor of ECM accumulation, both by decreasing the synthesis of ECM proteins and by increasing the degradation. In addition, Ets-1 in mesangial cells may inhibit TGF-β-mediated mesangial cell association with collagen fibers, thereby leading to abnormal ECM reorganization. The fact that the antifibrotic effect of Ets-1 was enhanced in the presence of TGF-β1 merits its therapeutic application into fibrosis. Therefore, *ets-1* gene transfer may be a feasible therapeutic tool not only to inhibit the accumulation of ECM proteins by blocking TGF-β signaling but also to promote degradation of ECM proteins by inducing MMP. In conclusion, Ets-1 maintains the plasticity of ECM proteins to be degraded and plays crucial roles for executing integral glomerular reorganization.

MATERIALS AND METHODS

Cell culture

Rat mesangial cells were isolated and cultured as described previously[41] and were maintained in RPMI 1640 (Sigma, St Louis, MO, USA) containing 20% fetal bovine serum equilibrated with 5% CO_2–95% air at 37°C. The cells at passage 6–8 were used. Mesangial cells were transfected with *ets-1* cDNA expression vector, pCNX2-m-ets-1S by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After incubation with RPMI 1640 containing 20% fetal bovine serum, G418 (final concentration; 800 μg/ml) (Sigma) was added to the medium for the selection of permanent transfectants. Established Ets-1 transfectants were maintained in RPMI 1640 with 20% fetal bovine serum and G418.

Electrophoretic mobility shift assay

Quiescent subconfluent normal mesangial cells or Ets-1 transfectants were treated with adding human recombinant TGF-β1 (1, 2, and 5 ng/ml) for 24 h. After incubation, cells were washed twice with ice-cold phosphate-buffered saline (PBS) without calcium and magnesium and scraped in 10 ml of cold PBS. Nuclear extracts were incubated with 100 ng/μl poly (d(I-C)) at room temperature for 5 min before adding the biotin-labeled transcription factor probe for Ets-1/PEA3 (Panomics, Inc., Redwood City, CA, USA) . Probe sequence was 5′-GATCTCGAGCAGGAAGTTCGA and non-labeled probe (cold probe) was used as control. The mixtures were separated on a non-denaturing polyacrylamide gel and electrotransferred to Biodyne B membrane (Pall, East Hills, NY, USA). After the immobilization of bound oligos using UV crosslinker, detection was performed with streproavidin–horseredish peroxidase conjugate.

Collagen gel contraction assay

Collagen gel contraction assays were performed to examine the effect of Ets-1 on mesangial cells to reorganize and contract three-dimensional collagen I gels.[21] Quiescent normal or Ets-1-transfectant mesangial cells were harvested at a concentration of 5×10^5 cells/ml in 0.5 ml of 1.25 × RPMI 1640 for 30 min at 20°C. The cell suspension was mixed on ice with 0.5 ml of collagen I solution (59% 1.25 × RPMI 1640, 40% rat tail collagen (3.75 mg/ml), 1% NaOH). Collagen/cell suspension (500 μl each) was incubated in 24-well plates (Costar, Cambridge, MA, USA) at 37°C to polymerize the collagen. The diameter of the hydrated gel was measured by use of an inverted microscope at 24 h. To examine the effect of Ets-1 on TGF-β-induced gel contraction, TGF-β1 (3.0 ng/ml) was added to collagen/cell suspension before gel polymerization.

Short synthetic interfering RNA

Short synthetic interfering RNA (21 nucleotides long) to target *ets-1* (siEts-1), and the scrambled genes (siSCR) were chemically synthesized as 2′ bis (acetoxyethoxy)-methyl ether-protected oligonucleotides, deprotected, annealed, and purified by Dharmacon Research (Lafayette, CO, USA). The sense and antisense strands of siEts-1 and siSCR were: siEts-1, 5′-ACGGAAGCGCAUCGAA GCCdTdT-3′ (sense), 5′-GGCUUCGAUGCGCUUCCGUdTdT-3′ (antisense); siSCR, 5′-GUCAACUGUGGAGCAACACdTdT-3′ (sense), 5′-GUGUUGCUCCACAGUUGACdTdT-3′ (antisense).

Northern blot analysis

We examined the effect of forced exogenous expression of Ets-1 on TGF-β-induced type I collagen expression *in vitro*. TGF-β-treated rat mesangial cells were rinsed twice with ice-cold PBS and scraped with 1 ml of Trizol reagent (Invitrogen). Total RNA was extracted according to the manufacturer's protocol. Type I collagen was labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Biosciences, Buckingham, England) by the random priming method (Rediprime II, Amersham Biosciences) and hybridization was carried out as described previously.[42]

Experimental design in anti-Thy-1 GN

To determine the effect of Ets-1 on mesangial proliferative glomerulonephritis, we employed an anti-Thy-1 model of glomerulonephritis. All procedure was handled in a humane fashion in accordance with the guidelines of the Animal Committee of Osaka University. Six-week-old male s.d. rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) and anti-Thy-1 model of glomerulonephritis was induced by an intravenous injection of anti-Thy-1 monoclonal antibody, 1-22-3 (5 μg/kg).[43] On day 3, the kidney was perfused with PBS via renal artery and then *ets-1* expression vector, pCNX2-m-ets-1S (200 μg in 0.5 ml of PBS), siEts-1 (50 μg), or siSCR (50 μg) was injected into left kidney in a one shot manner (six rats in each group). The kidney was sandwiched with tweezers-type of oval-shaped stainless electrode, and electric pulses were delivered using an electric pulse generator (Electro Square Porator T820; BTX, San Diago, CA, USA). The shape of the pulse was a square wave and the voltage (75 V) remained constant during the pulse duration. Three pulses of the indicated voltage followed by three more pulses of the opposite polarity were administrated to the kidney. Intra-pulse delay was 1 s and the duration of the pulse was fixed at 100 ms.[41]

On day 7, treated left kidneys and untreated contralateral right kidneys were perfused with cold autoclaved PBS, and samples of tissues for light microscopy were fixed with 4% paraformaldehyde overnight and dehydrated through a graded ethanol series and embedded in paraffin. Histological sections (2 μm) of the kidneys were stained with periodic acid-Schiff reagent. For glomerular RNA preparations, glomeruli were isolated from the pooled remaining renal tissue by a standard sieving method.

Pathological changes in anti-Thy-1 nephritis model are heterogeneous among different glomeruli. Histological improvement of the disease manifestations was quantitatively estimated. For each samples, approximately 30 cortical glomerular cross-sections containing vascular pole were evaluated in a blind fashion. Glomerular

ECM deposition was graded semiquantitatively according to the score described elsewhere. It ranged from 0 to 4; 0 = glomerulus without any deposition, 1 = glomerulus with up to 25% deposition, 2 = glomerulus with 26–50% deposition, 3 = glomerulus with 51–75% deposition, 4 = glomerulus with 76–100% deposition. Glomerular expression of SMαA, fibronection EDA, and type I collagen was evaluated by computerized image analysis using Mac SCOPE program. In brief, selected glomeruli from each sample were photographed and positive areas were highlighted on the captured images. The area of positive staining relative to each glomerular area was automatically calculated as a percentage with determined threshold settings.

Immunohistochemistry

To examine the expression of Ets-1 and SMαA, immunohistochemistry staining was performed on tissues fixed in methyl Carnoy's solution. Tissue sections were preincubated with horse serum diluted 1:20 with PBS for 30 min to block the nonspecific staining, and then incubated with rabbit polyclonal antibody against Ets-1 (N-276, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or mouse immunoglobulin G anti-SMαA monoclonal antibodies (Immunotech S.A., Cedex, Marseilles, France) for 60 min at room temperature. The sections were then processed using an avidin-biotinylated peroxidase complex method (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA) with diaminobenzidine as the chromogen.

Treated kidneys were also examined by immunofluorescence. Kidneys were fixed in ice-cold neutral-buffered 4% paraformaldehyde solution and embedded in O.C.T. compound (Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA, USA). The cryostat sections were stained with the monoclonal antibody against fibronectin EDA (IST-9, Abcam, Cambridge, UK) and type I collagen (LSL Co., Tokyo, Japan). After blocking in 5% normal horse serum, sections were incubated with first antibody for 1 h at room temperature, followed by Texas red-conjugated anti-mouse immunoglobulin G (1:200) (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature.

Reverse transcriptase-PCR

We examined the glomerular ets-1 and MMP mRNA expression during the disease progression of anti-Thy-1 nephritis and investigated the effect of forced exogenous expression of Ets-1 on MMP expression in nephritic glomeruli. Isolated glomeruli from Ets-1-transfected nephritic kidneys or untreated contralateral right kidneys were homogenized with Polytron homogenizer (Kinematica, Switzerland) in Trizol reagent after Ets-1 transfection. Total RNA was extracted according to the manufacturer's protocol. RNA was denatured by heating and reverse-transcribed with reverse transcriptase. PCR was performed in the DNA thermal cycler (Applied Biosystems, Foster City, CA, USA) with 100 μg of reaction mixture and 5 U of *Taq* DNA polymerase. PCR products were run through 1% agarose gels.

The following primer pairs were synthesized and used for amplification: for exogenously introduced ets-1, sense 5′-TCTGACTGACCGTACTC-3′ and antisense 5′-GGGTTGGCTCCATTAACCTG-3′; for MMP-1, sense 5′-TCTGACTGACCGTACTC-3′ and antisense 5′-GGGTTGGCTCCATTAACCTG-3′; for MMP-2, sense 5′-TCTGACTGACCGTACTC-3′ and antisense 5′-GGGTTGGCTCCATTAACCTG-3′; for MMP-3, sense 5′-TCTGACTGACCGTACTC-3′ and antisense 5′-GGGTTGGCTCCATTAACCTG-3′; for MMP-9, sense 5′-TCTGACTGACCGTACTC-3′ and antisense 5′-GGGTTGGCTCCATTAACCTG-3′; for tissue inhibitor of metalloproteinase-1, sense 5′-TCTGACTGACCGTACTC-3′ and antisense 5′-GGGTTGGCTCCATTAACCTG-3′.

Zymography

We also examined the effect of forced exogenous expression of Ets-1 on MMP activities in nephritic glomeruli by zymography. Collected glomeruli were homogenized in 1 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet-P40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mM sodium orthovanadate by Polytoron homogenizer at 4°C. The soluble lysates were suspended in non-reducing sample buffer, electrophoresed on a 7.5% sodium dodecyl sulfate-polyacrylamide gel containing 2 mg/ml gelatin, washed in renaturating buffer (50 nM Tris/HCl (pH 8.0), 2.5% Triton X-100), and incubated overnight at 37°C in 50 nM Tris/HCl (pH 8.0), 1 μM $ZnSO_4$, 5 nM $CaCl_2$. Gels were methanol-fixed, stained with Coomassie blue R-250 before destaining.

Western blot

To confirm the successful transfection of *Ets-1* gene or examine the effect of forced exogenous expression of Ets-1 on fibronectin EDA and type I collagen expression *in vivo*, Western blot analysis was performed. Collected glomeruli were homogenized in 1 ml of lysis buffer at 4°C. The soluble lysates were mixed 1:2 with 3 × Laemmli buffer and heated for 10 min at 95°C. Lysates (20 μg) were loaded per lane, resolved by 15% sodium dodecyl sulfate-PAGE and transferred onto a polyvinylidene difluoride membrane (Hybond-P PVDF Membrane, Amersham Biosciences). Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline–Tween 20 for 30 min at room temperature and then immunoblotted with polyclonal antibodies against Ets-1 (N-27, Santa Cruz Biotechnology, Santa Cruz, CA, USA), fibronectin EDA (LSL Co, Tokyo, Japan), and type I collagen (IST-9, abcam, Cambridge, UK), in blocking buffer for 1 h at room temperature. The primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit and visualized with SuperSignal West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL, USA) according to the manufacturer's directions. The blots were exposed to X-ray film (Hyperfilm, Amersham Biosciences).

Statistical study

All values are expressed as means ± s.d. Statistical significance (defined as $P < 0.01$) was evaluated using the one-way analysis of variance.

REFERENCES

1. Ishimura E, Sterzel RB, Budde K *et al.* Formation of extracellular matrix by cultured rat mesangial cells. *Am J Pathol* 1989; **134**: 843–855.
2. Natori Y, O'Meara YM, Manning EC *et al.* Production and polarized secretion of basement membrane components by glomerular epithelial cells. *Am J Physiol* 1992; **262**: F131–F137.
3. Norman JT, Fine LG. Progressive renal disease: fibroblasts, extracellular matrix, and integrins. *Exp Nephrol* 1999; **7**: 167–177.
4. Yoshioka K, Tohda M, Takemura T *et al.* Distribution of type I collagen in human kidney diseases in comparison with type III collagen. *J Pathol* 1990; **162**: 141–148.
5. Eddy AA. Molecular basis of renal fibrosis. *Pediatr Nephrol* 2000; **15**: 290–301.
6. Kola I, Brookes S, Green AR *et al.* The Ets1 transcription factor is widely expressed during murine embryo development and is associated with

mesodermal cells involved in morphogenetic processes such as organ formation. *Proc Natl Acad Sci USA* 1993; **90**: 7588–7592.
7. Oda N, Abe M, Sato Y. ETS-1 converts endothelial cells to the angiogenic phenotype by inducing the expression of matrix metalloproteinases and integrin beta3. *J Cell Physiol* 1999; **178**: 121–132.
8. Reisdorff J, En-Nia A, Stefanidis I *et al.* Transcription factor Ets-1 regulates gelatinase a gene expression in mesangial cells. *J Am Soc Nephrol* 2002; **13**: 1568–1578.
9. Vandenbunder B, Pardanaud L, Jaffredo T *et al.* Complementary patterns of expression of c-ets 1, c-myb and c-myc in the blood-forming system of the chick embryo. *Development* 1989; **107**: 265–274.
10. Wasylyk B, Wasylyk C, Flores P *et al.* The c-ets proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. *Nature* 1990; **346**: 191–193.
11. Gum R, Lengyel E, Juarez J *et al.* Stimulation of 92-kDa gelatinase B promoter activity by ras is mitogen-activated protein kinase kinase 1-independent and requires multiple transcription factor binding sites including closely spaced PEA3/ets and AP-1 sequences. *J Biol Chem* 1996; **271**: 10672–10680.
12. Gutman A, Wasylyk B. The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites. *EMBO J* 1990; **9**: 2241–2246.
13. Iwasaka C, Tanaka K, Abe M *et al.* Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metalloproteinase-1 and the migration of vascular endothelial cells. *J Cell Physiol* 1996; **169**: 522–531.
14. Logan SK, Garabedian MJ, Campbell CE *et al.* Synergistic transcriptional activation of the tissue inhibitor of metalloproteinases-1 promoter via functional interaction of AP-1 and Ets-1 transcription factors. *J Biol Chem* 1996; **271**: 774–782.
15. Nerlov C, De Cesare D, Pergola F *et al.* A regulatory element that mediates co-operation between a PEA3-AP-1 element and an AP-1 site is required for phorbol ester induction of urokinase enhancer activity in HepG2 hepatoma cells. *EMBO J* 1992; **11**: 4573–4582.
16. Wasylyk C, Gutman A, Nicholson R *et al.* The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. *EMBO J* 1991; **10**: 1127–1134.
17. Sato Y. Transcription factor ETS-1 as a molecular target for angiogenesis inhibition. *Hum Cell* 1998; **11**: 207–214.
18. Cederberg A, Hulander M, Carlsson P *et al.* The kidney-expressed winged helix transcription factor FREAC-4 is regulated by Ets-1. A possible role in kidney development. *J Biol Chem* 1999; **274**: 165–169.
19. Naito T, Razzaque MS, Nazneen A *et al.* Renal expression of the Ets-1 proto-oncogene during progression of rat crescentic glomerulonephritis. *J Am Soc Nephrol* 2000; **11**: 2243–2255.
20. Raffetseder U, Wernert N, Ostendorf T *et al.* Mesangial cell expression of proto-oncogene Ets-1 during progression of mesangioproliferative glomerulonephritis. *Kidney Int* 2004; **66**: 622–632.
21. Kagami S, Kondo S, Loster K *et al.* Alpha1beta1 integrin-mediated collagen matrix remodeling by rat mesangial cells is differentially regulated by transforming growth factor-beta and platelet-derived growth factor-BB. *J Am Soc Nephrol* 1999; **10**: 779–789.
22. Tsujie M, Isaka Y, Nakamura H *et al.* Electroporation-mediated gene transfer that targets glomeruli. *J Am Soc Nephrol* 2001; **12**: 949–954.
23. Border W, Noble N. Transforming growth factor in tissue fibrosis. *N Engl J Med* 1994; **331**: 1286–1292.
24. Douthwaite JA, Johnson TS, Haylor JL *et al.* Effects of transforming growth factor-beta1 on renal extracellular matrix components and their regulating proteins. *J Am Soc Nephrol* 1999; **10**: 2109–2119.
25. Basuyaux JP, Ferreira E, Stehelin D *et al.* The Ets transcription factors interact with each other and with the c-Fos/c-Jun complex via distinct protein domains in a DNA-dependent and -independent manner. *J Biol Chem* 1997; **272**: 26188–26195.
26. Czuwara-Ladykowska J, Sementchenko VI, Watson DK *et al.* Ets1 is an effector of the transforming growth factor beta (TGF-beta) signaling pathway and an antagonist of the profibrotic effects of TGF-beta. *J Biol Chem* 2002; **277**: 20399–20408.
27. Racine-Samson L, Rockey DC, Bissell DM. The role of alpha1beta1 integrin in wound contraction. A quantitative analysis of liver myofibroblasts *in vivo* and in primary culture. *J Biol Chem* 1997; **272**: 30911–30917.
28. Nunes I, Gleizes PE, Metz CN *et al.* Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta. *J Cell Biol* 1997; **136**: 1151–1163.
29. Johnson TS, Skill NJ, El Nahas AM *et al.* Transglutaminase transcription and antigen translocation in experimental renal scarring. *J Am Soc Nephrol* 1999; **10**: 2146–2157.
30. Tomita N, Morishita R, Taniyama Y *et al.* Angiogenic property of hepatocyte growth factor is dependent on upregulation of essential transcription factor for angiogenesis, ets-1. *Circulation* 2003; **107**: 1411–1417.
31. Chernavvsky DR, Fernandez LG, Barton K *et al.* Expression and function of the ETS-1 gene in the developing kidney [Abstract]. *J Am Soc Nephrol* 1998; **9**: 360A.
32. Okuda S, Languino LR, Ruoslahti E *et al.* Elevated expression of transforming growth factor-beta and proteoglycan production in experimental glomerulonephritis. Possible role in expansion of the mesangial extracellular matrix. *J Clin Invest* 1990; **86**: 453–462.
33. Tomooka S, Border WA, Marshall BC *et al.* Glomerular matrix accumulation is linked to inhibition of the plasmin protease system. *Kidney Int* 1992; **42**: 1462–1469.
34. Kagami S, Border WA, Ruoslahti E *et al.* Coordinated expression of beta 1 integrins and transforming growth factor-beta-induced matrix proteins in glomerulonephritis. *Lab Invest* 1993; **69**: 68–76.
35. Tanaka K, Oda N, Iwasaka C *et al.* Induction of Ets-1 in endothelial cells during reendothelialization after denuding injury. *J Cell Physiol* 1998; **176**: 235–244.
36. Sato Y, Kanno S, Oda N *et al.* Properties of two VEGF receptors, Flt-1 and KDR, in signal transduction. *Ann N Y Acad Sci* 2000; **902**: 201–205; discussion 205–207.
37. Hugo C, Shankland SJ, Bowen-Pope DF *et al.* Extraglomerular origin of the mesangial cell after injury. A new role of the juxtaglomerular apparatus. *J Clin Invest* 1997; **100**: 786–794.
38. Masuda Y, Shimizu A, Mori T *et al.* Vascular endothelial growth factor enhances glomerular capillary repair and accelerates resolution of experimentally induced glomerulonephritis. *Am J Pathol* 2001; **159**: 599–608.
39. Mizuno S, Kurosawa T, Matsumoto K *et al.* Hepatocyte growth factor prevents renal fibrosis and dysfunction in a mouse model of chronic renal disease. *J Clin Invest* 1998; **101**: 1827–1834.
40. Teruyama K, Abe M, Nakano T *et al.* Role of transcription factor Ets-1 in the apoptosis of human vascular endothelial cells. *J Cell Physiol* 2001; **188**: 243–252.
41. Isaka Y, Nakamura H, Mizui M *et al.* DNAzyme for TGF-beta suppressed extracellular matrix accumulation in experimental glomerulonephritis. *Kidney Int* 2004; **66**: 586–590.
42. Nakamura H, Isaka Y, Tsujie M *et al.* Introduction of DNA enzyme for Egr-1 into tubulointerstitial fibroblasts by electroporation reduced interstitial alpha-smooth muscle actin expression and fibrosis in unilateral ureteral obstruction (UUO) rats. *Gene Therapy* 2002; **9**: 495–502.
43. Kawachi H, Orikasa M, Matsui K *et al.* Epitope-specific induction of mesangial lesions with proteinuria by a MoAb against mesangial cell surface antigen. *Clin Exp Immunol* 1992; **88**: 399–404.